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Transcription Factor STAT3 and Type I Interferons Are Corepressive Insulators for Differentiation of Follicular Helper and T Helper 1 Cells

John P. Ray,[1] Heather D. Marshall,[1] Brian J. Laidlaw,[1] Matthew M. Staron,[1] Susan M. Kaech,[1,2] and Joe Craft[1,3,*]
[1]Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06520, USA
[2]Howard Hughes Medical Institute, 4000 Jones Bridge Road, Chevy Chase, MD 20815-6789, USA
[3]Department of Medicine (Rheumatology), Yale University School of Medicine, New Haven, CT 06520, USA
*Correspondence: joseph.craft@yale.edu
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SUMMARY

Follicular helper T (Tfh) cells are required for the establishment of T-dependent B cell memory and high affinity antibody-secreting cells. We have revealed herein opposing roles for signal transducer and activator of transcription 3 (STAT3) and type I interferon (IFN) signaling in the differentiation of Tfh cells following viral infection. STAT3-deficient CD4$^+$ T cells had a profound defect in Tfh cell differentiation, accompanied by decreased germinal center (GC) B cells and antigen-specific antibody production during acute infection with lymphocytic choriomeningitis virus. STAT3-deficient Tfh cells had strikingly increased expression of a number of IFN-inducible genes, in addition to enhanced T-bet synthesis, thus adopting a T helper 1 (Th1) cell-like effector phenotype. Conversely, IFN-αβ receptor blockade restored Tfh and GC B cell phenotypes in mice containing STAT3-deficient CD4$^+$ T cells. These data suggest mutually repressive roles for STAT3 and type I IFN signaling pathways in the differentiation of Tfh cells following viral infection.

INTRODUCTION

Follicular T helper (Tfh) cells are a subset of CD4$^+$ T cells required for the T-dependent germinal center (GC) response leading to the production of antigen-specific memory B and plasma cells (Crotty, 2011; McHeyzer-Williams et al., 2012). Proper regulation of Tfh cell differentiation in secondary lymphoid organs (SLOs) is critical for controlled immune function. Poor response of these cells is associated with a defective GC reaction (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009), whereas their overabundance can lead to pathogenic autoantibody production and autoimmune disease (Linterman et al., 2009; Vinuesa et al., 2005). Upregulation of B cell lymphoma 6 (Bcl6), the canonical Tfh cell transcription factor, and downregulation of its transcriptional repressor B-lymphocyte-induced maturation protein 1 (Blimp-1), are required for initiation of the Tfh cell development program (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). Expression of Bcl6, concomitant with downregulation of the chemokine receptor CCR7 and P-selectin glycoprotein ligand-1 (PGSL-1) in concert with CXCR5 upregulation, enables Tfh cells to emigrate from the T cell zone of SLOs to the B cell follicle where they can promote GC reactions (Haynes et al., 2007; Marshall et al., 2011; Poholek et al., 2010). Bcl6 upregulation in nascent Tfh cells occurs in a two-step process dependent upon inducible T cell costimulator (ICOS) signaling via ICOS-ligand (ICOS-L), delivered first by dendritic cells in the T cell zone of SLOs, and second by interactions with B cells at the T-B border in the spleen and interfollicular regions of lymph nodes (Choi et al., 2013; Coffey et al., 2009; Kerfoot et al., 2011).

Previous work has suggested a role for the inflammatory milieu in promoting the Tfh cell phenotype, particularly those cytokines that are known to signal through signal transducer and activator of transcription 3 (STAT3). For example, the cytokines interleukin-6 (IL-6), IL-21, and IL-27 have been implicated in Tfh cell development, albeit with differing roles. IL-6 is required for development of Tfh cells early following viral challenge (Choi et al., 2013), while also promoting their maintenance later in chronic viral infections (Harker et al., 2011), with IL-27 needed for their maintenance upon protein immunization (Batten et al., 2010). IL-21 has also been reported to be important for Tfh cell differentiation (Nurieva et al., 2008; Vogelzang et al., 2008), although such a role has not been universally found, a difference perhaps reflecting mode of immunization (Linterman et al., 2010; Zotos et al., 2010). In the absence of IL-6, IL-21 is more important in later stages following protein immunization or viral challenge (Eto et al., 2011; Karnowski et al., 2012), yet it is not required early in Tfh cell differentiation (Choi et al., 2013). As would be expected from these results, STAT3 has been reported to be required for the development of CXCR5$^+$ CD4$^+$ T cells, following challenge with the antigen KLH in complete Freund's adjuvant and their subsequent function in promoting the development of peanut agglutinin$^+$ (PNA$^+$) GC B cells (Nurieva et al., 2008). Human subjects with dominant-negative mutations in STAT3 also display reduced numbers of CXCR5$^+$ circulating CD4$^+$ T cells, related to Tfh cells in SLOs further suggesting the potential importance of this signaling pathway in Tfh cell differentiation (Ma et al., 2012). Yet, work using adoptive transfers of viral-specific T cell receptor (TCR) transgenic CD4$^+$ T cells reported a requirement for STAT3 in Tfh cell development only within the first 48 hr following viral infection, with normal Tfh cell differentiation ensuing by 3 days after infection (Choi et al., 2013). This

finding is inconsistent with the broader roles of STAT3 cytokines in Tfh cell development and maintenance.

Here, we have demonstrated a critical role for STAT3 in Tfh cell development and function following acute viral infection. STAT3 expression in $CD4^+$ T cells is required for their differentiation into Tfh cells and promotion of GC B cell development and virus-specific antibody responses. We also identify a role for STAT3 in downmodulating type I interferon (IFN) signaling, as STAT3-deficient Tfh cells display a marked increase in Th1 cell-associated and IFN-inducible transcripts. Accordingly, suppression of type I IFN signaling by antibody blockade of the IFN-αβ receptor promoted Tfh cell differentiation in wild-type (WT) mice and mice containing STAT3-deficient $CD4^+$ T cells. The treatment also rescued the GC and pathogen-specific antibody defect found in the STAT3 mutant mice. This effect was specific to type I IFNs, as blockade of IFN-γ did not substantially alter Tfh cell percentages, nor affected GC B cell percentages after infection. These findings demonstrate the importance of STAT3 and the STAT3-activating cytokines in promoting Tfh cell differentiation and function during viral infection, and furthermore, reveal contrasting roles for STAT3 and type I IFNs in determining the balance between Tfh and Th1 cell differentiation.

RESULTS

Mice Containing STAT3-Deficient $CD4^+$ T Cells Have a Reduction in Tfh Cell Differentiation and Impaired GC Formation

Previous work using an antigen-adjuvant system demonstrated that STAT3 has a T cell-intrinsic effect on the development of $CD4^+$ $CXCR5^{hi}$ cells and the promotion of GC B cell differentiation (Nurieva et al., 2008). However, its role in Tfh cell development and function following viral infection appears limited to the first 2 days following challenge (Choi et al., 2013), an unexpected finding given that multiple STAT3-dependent cytokines have been implicated in Tfh cell differentiation and continue to be produced after day 3 of infection (Batten et al., 2010; Eto et al., 2011). To address this issue, we ablated STAT3 from all $CD4^+$ T cells by breeding $Stat3^{fl/fl}$ mice to $Cd4^{cre}$ mice ($Stat3^{fl/fl}Cd4^{cre}$ mice; abbreviated $Stat3^{-/-}$ in the Figures) (Schmidt-Supprian and Rajewsky, 2007; Welte et al., 2003; Wolfer et al., 2001), and infected them with the Armstrong strain of lymphocytic choriomeningitis virus (LCMV). $Stat3^{fl/fl}$ *cre*-negative littermates were used as controls and are designated as WT for simplicity. At 8 days postinfection (dpi) we observed similar frequencies of LCMV GP_{66}-specific $CD4^+$ $CD44^+$ T cells in both $Stat3^{fl/fl}Cd4^{cre}$ and WT littermate control mice despite differences in splenocyte numbers (see Figures S1A and S1B available online). Next, we compared the phenotypes of $CD4^+$ Th1 and Tfh cells that formed in $Stat3^{fl/fl}Cd4^{cre}$ and WT $CD4^+$ T cells that have been previously characterized during LCMV infection (Hale et al., 2013; Marshall et al., 2011). Increased expression of PSGL-1 and Ly6C and decreased expression of the chemokine receptor CXCR5 helped to distinguish Th1 cells that expressed high amounts of T-bet, IFN-γ, and granzyme B and were localized in the red pulp (Figure 1A, red gates). Another subset of PSGL-1^{hi} $Ly6C^{lo}$ cells were mostly localized in the T cell zone and expressed lower amounts of T-bet and IFN-γ; compared to the PSGL-1^{hi} $Ly6C^{hi}$ cells, some of these cells also expressed CXCR5 (Figure 1A, blue gates) (Hale et al., 2013; Marshall et al., 2011). Conversely, decreased expression of Ly6C and PSGL-1 and increased expression of CXCR5 distinguished Tfh cells (Figures 1A; see Figure S1A available online, green gates) (Hale et al., 2013; Marshall et al., 2011). Notably, all PSGL-1^{lo} cells expressed CXCR5, supporting previous evidence that these cells are follicular T cells (Figure S1A) (Poholek et al., 2010). Compared to the WT $CD44^+$ or GP_{66}-specific $CD4^+$ T cells, the $Stat3^{fl/fl}Cd4^{cre}$ cells displayed a reduction in the proportion and numbers of GP_{66}^+ and polyclonal $Ly6c^{lo}$ PSGL-1^{lo} cells and $Ly6c^{lo}$ PSGL-1^{lo} $CXCR5^{hi}$ Tfh cells at 8 dpi (Figures 1A–1E; Figures S1A and S1C–S1E). The mean fluorescence intensities (MFI) of CXCR5 in the polyclonal and GP_{66}^+ $Ly6c^{lo}$ PSGL-1^{lo} populations were also reduced in cells from the $Stat3^{fl/fl}Cd4^{cre}$ mice compared to WT littermates (Figures S1F and S1G). In association with a reduction in $Ly6c^{lo}$ PSGL-1^{lo} $CXCR5^{hi}$ Tfh cells in $Stat3^{fl/fl}Cd4^{cre}$ mice, GC B cells observed by flow cytometry were considerably reduced at 8 dpi compared to the WT mice (Figures 1F–1H). These findings were further corroborated by confocal microscopy, which revealed that mice with STAT3-deficient $CD4^+$ T cells have fewer GCs per B cell follicle (Figures 1I and 1J). The large reduction in GC B cells seemed disproportionate to the diminution of Tfh cells, so we analyzed the deletion efficiency of *Stat3* from $CD4^+$ T cells versus B cells, finding 97% deletion in $CD4^+$ T cells, with none in B cells (Figure S1H). A similar impairment in Tfh cell differentiation and GC B cell responses was observed at 14 dpi (Figures S2A–S2H). Thus, STAT3 activity in $CD4^+$ T cells is critical for formation of Tfh cells and activation of GC responses during viral infection.

$Stat3^{fl/fl}Cd4^{cre}$ Mice Fail to Promote Robust Isotype-Switched LCMV-Specific Antibody Production

With the defect in GC formation in the $Stat3^{fl/fl}Cd4^{cre}$ mice, we wondered whether early plasmablast responses were also affected. These occur in extrafollicular regions of SLOs, before highly affinity matured and isotype switched B cells exit the GC (Zotos et al., 2010). The plasmablast response to LCMV infection was analyzed with ELISAs to measure the generation of antigen-specific antibodies in the serum at 8 dpi. We found that there was a significant decrease in LCMV-specific immunoglobulin M (IgM) and IgG in the $Stat3^{fl/fl}Cd4^{cre}$ mice, compared to WT animals (Figures 2A and 2B). By day 14 dpi, IgM responses were rescued, whereas IgG responses remained hindered, suggesting that LCMV-specific plasmablasts and plasma cells were either delayed or reduced in class-switch recombination to IgG (Figures 2C and 2D). We also found that IgG2a antibodies were decreased at 14 dpi, although this decrease was not significant (Figure 2E). These data show there is a significant defect in the IgG antibody response following LCMV infection of $Stat3^{fl/fl}Cd4^{cre}$ mice.

STAT3-Deficient Tfh Cells Phenotypically Resemble Th1 Cells

We next examined the effects of STAT3-deficiency on Tfh cell properties such as Bcl6 expression and IL-21 production, a Tfh cell-producing cytokine that promotes GC longevity, B cell survival (Linterman et al., 2010; Zotos et al., 2010), and class switch recombination to IgG1 (Ozaki et al., 2002). Bcl6 protein was significantly decreased at 5 dpi (Figures 3A and 3B); however, we found no differences at 8 dpi (data not shown). We also

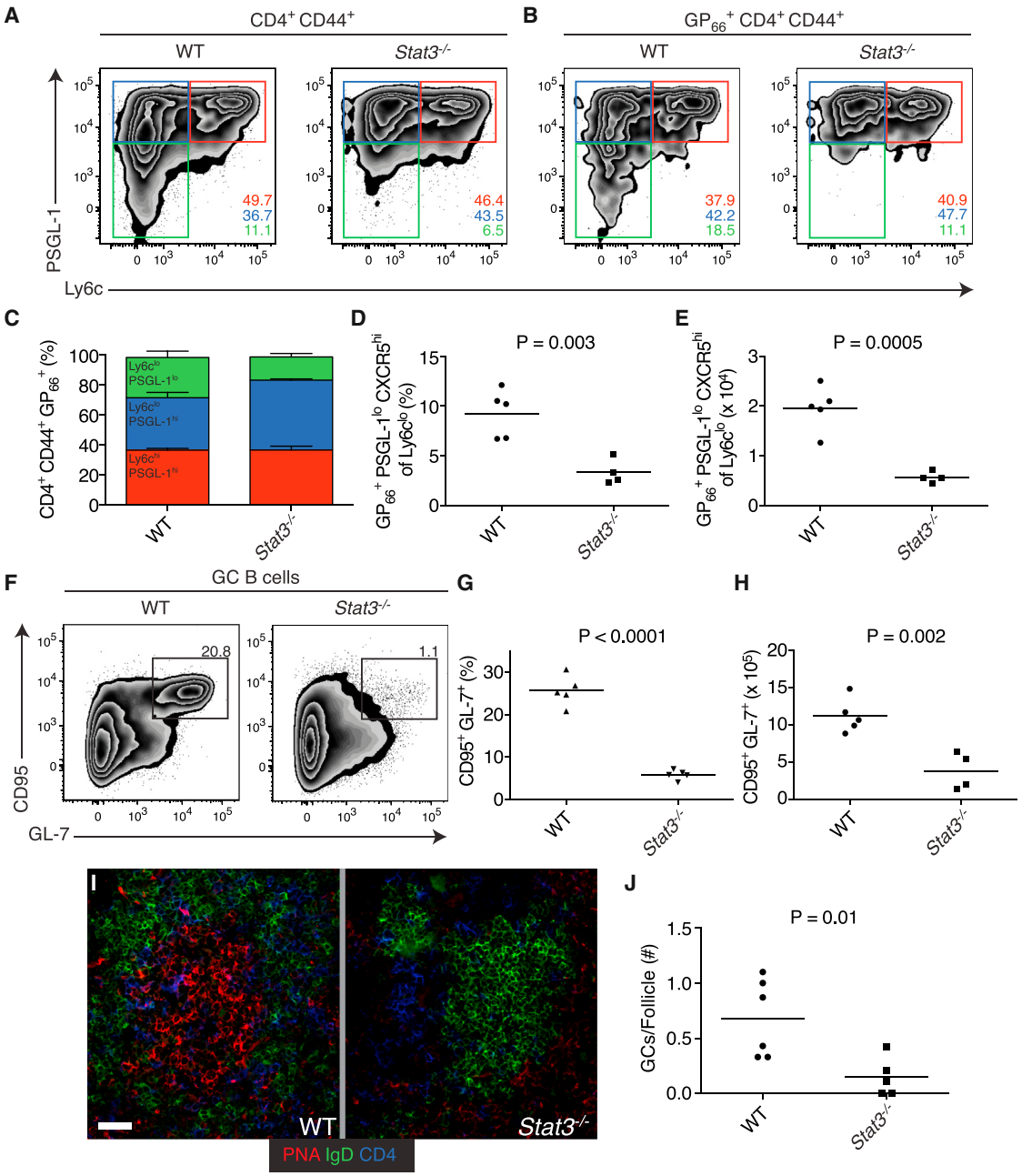


Figure 1. *Stat3*$^{fl/fl}$*Cd4*^{cre} Mice Have an Impaired Tfh Cell Response following Acute Viral Infection

(A and B) Flow cytometry plots of CD44$^+$ (A) and GP$_{66}^+$ LCMV epitope-specific (B) CD4$^+$CD44$^+$ T cells gated on Ly6c and PSGL-1. Th1 terminal effector precursors are Ly6c^{hi} PSGL-1^{hi} cells (red), Th1 central memory precursors are Ly6c^{lo} PSGL-1^{hi} (blue), and Tfh cells are Ly6c^{lo} PSGL-1^{lo} (green).

(C) Population distribution of terminal effector precursors, central memory precursors, and Tfh cells based on Ly6c and PSGL-1, gating as in (A).

(D and E) GP$_{66}$-specific Tfh cell percentages (D) and numbers (E), gated as Ly6c^{lo} PSGL-1^{lo} CXCR5^{hi}.

(F–H) Flow cytometry plots (F), percentages (G), and numbers (H) of GC B cells, gated as B220$^+$ IgD^{lo} CD95^{hi} GL-7$^+$.

(I) Immunohistochemistry of spleen sections, identifying GCs with anti-PNA (red), anti-IgD (green), and anti-CD4 (blue). Scale bar represents 25 μm.

(J) Quantification of GCs per B cell follicle.

(A–H) Representative of at least four experiments with at least four animals per genotype per experiment. + SEM (C).

(I and J) Representative of sections from at least five animals of each genotype. Statistics conducted with Student's unpaired t test. WT, wild-type (*Stat3*$^{fl/fl}$ *cre*-negative) mice. See also Figure S1.

observed a decrease in the percentages of CD4$^+$ CD44$^+$ CXCR5^{hi} IL-21 and IFN-γ double producers (IL-21 IFN-γ DP) at 8 dpi (Figures 3C and 3D). This trend was shared with IL-21-single producers (IL-21 SP), but there was no difference in IFN-γ SP between *Stat3*$^{fl/fl}$ *cre*-negative WT and mutant mice (Figures 3E and 3F).

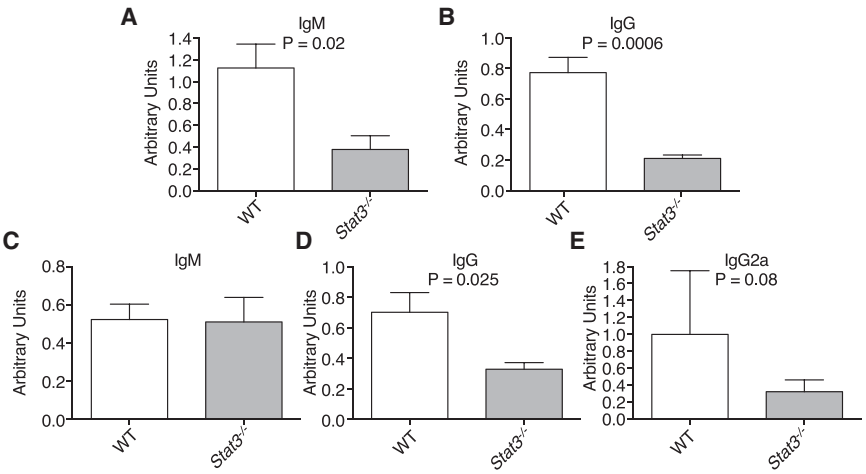


Figure 2. *Stat3*$^{fl/fl}$*Cd4*^{cre} (*Stat3*$^{-/-}$) Mice Are Hindered in their Ability to Create LCMV-Specific Antibodies

(A and B) IgM and IgG anti-LCMV at 8 dpi.
(C–E) IgM, IgG, and IgG2a anti-LCMV at 14 dpi.
(A–E) Representative of at least two experiments with at least four animals per genotype per experiment. Statistics conducted using Student's unpaired t test. + SEM (A–D). See also Figure S2.

Repression of Tfh cell differentiation following viral infection has been shown to occur through IL-2-mediated STAT5 signaling via the high-affinity IL-2Rα chain (CD25), with promotion of Blimp-1 expression and suppression of Bcl6 (Choi et al., 2011; Johnston et al., 2012; Nurieva et al., 2012; Pepper et al., 2011). Accompanying the decrease in Bcl6 in the STAT3-deficient CD4$^+$ T cells, we found increased CD25 expression at 5 dpi, a time at which WT Tfh cells normally turn down expression of this receptor (Figures 3G and 3H). We also found an increase in T-bet expression at this time point and at 14 dpi (Figures 3I and 3J; Figures S2I and S2J), with no difference at 8 dpi (data not shown), in line with the increase in Th1 cell development. These data show that STAT3 promotes Tfh cell differentiation in part by inducing Bcl6 and suppressing T-bet and IL-2 signaling.

Gene-Expression Analysis Reveals a STAT1-Driven Transcriptome in STAT3-Deficient Tfh Cells

Because STAT3-deficient Tfh cells had characteristics of Th1 cells, we next asked whether their global gene expression was also consistent with that phenotype. Therefore, we performed RNA-sequencing (RNA-seq) on sorted polyclonal CD4$^+$CD44$^+$ T cells from WT and STAT3-deficient CD4$^+$ T cells, again by using Ly6c and PSGL-1 expression to distinguish the three effector populations, with further gating on the CXCR5^{hi} group within the Ly6c^{lo} PSGL-1^{lo} subset to distinguish Tfh cells (Figure 1A; Figure S1A). We used a multidimensional scaling plot to relate the similarities and differences among the populations relative to one another (Figure S3A), finding that *Stat3*$^{fl/fl}$ *cre*-negative WT Tfh cells (Ly6c^{lo} PSGL-1^{lo} CXCR5^{hi}) had vastly different gene expression than either their Th1 Ly6c^{lo} PSGL-1^{hi} or Th1 Ly6c^{hi} PSGL-1^{hi} counterparts, validating their separation by surface marker expression (Marshall et al., 2011). In addition, we used unbiased K-means clustering to determine how global STAT3-deficient Tfh cell gene expression compares to the three WT populations (Figure 4A; Figure S3B). We found that STAT3-deficient Tfh cells had a transcriptome more similar to that of WT Th1 Ly6c^{lo} PSGL-1^{hi} cells than the WT Tfh cells, as determined by dendrogram (Figure 4A) and expression patterns (Figure 4A; Figure S3B). Integrated Pathway Analysis (IPA, Ingenuity® Systems, www.ingenuity.com) revealed that helper T cell differentiation was the most deregulated pathway in STAT3-deficient Tfh cells with upregulation of genes associated with T helper cell lineages such as regulatory T cells (i.e., *Foxp3* and *Il10*) (Figure 4B; Figure S3C). To corroborate our findings that STAT3-deficient Tfh cells were phenotypically similar to Th1 cells, we also found upregulated expression of genes associated with this lineage (i.e., *Tbx21*, *Ifngr1*, *Il12r*, and *Il2ra*) (Figure 4B). In juxtaposition, six out of seven genes with decreased expression in this canonical pathway were Tfh-cell associated (*Bcl6*, *Il21*, *Il4*, *Icos*, *Cxcr5*, and *Il6st*) (Figure 4B). In accordance with the downregulation of *Bcl6* and other Tfh-cell associated transcripts, we found an increase in *Prdm1*, which encodes Blimp-1 (Figure 4C). We validated *Bcl6*, *Il21*, *Il4*, and *Prdm1* expression by qPCR (Figures S4A–S4D). Also in line with our RNA-seq data, we observed an increase in Foxp3$^+$ CD44$^+$ Ly6c^{lo} CXCR5^{hi} follicular regulatory T (Tfr) cells, with no differences in GP$_{66}^+$ Tfr cells at 8 dpi, although these cells seem to be rather uncommon at this time point in LCMV infection (Figures S4E and S4F).

STAT3-deficient Tfh cells also had a marked increase in RNA expression of IFN-inducible genes over their WT littermates (Figure 4C), including transcripts downstream of both type I and type II IFN signal transduction (Figure 4C; Figure S4G). We then determined potential upstream regulators by using IPA analysis, finding that IFN regulatory factor-7 (IRF7) and STAT1 were the top two transcription factors affiliated with the changes in gene expression between STAT3-deficient versus WT Tfh cells (Table S1). We also found suppressor of cytokine signaling-1 (SOCS1) and SOCS3 phosphatase activity to be potentially decreased in the STAT3-deficient Tfh cells based on the IPA analysis (Table S1). As both SOCS1 and SOCS3 can act as repressors of STAT1 (Song and Shuai, 1998), an abundance of STAT1-mediated transcription might occur due to this mechanism in the absence of STAT3. However, SOCS1 and SOCS3 were not substantially reduced in the absence of STAT3 according to our RNA-seq results (Figure 4C), and upon further analysis with qPCR, we found that these mRNAs were actually upregulated in cells from *Stat3*$^{fl/fl}$*Cd4*^{cre} animals (Figures S4H and S4I), a phenomenon that might be due to an increase in type I IFN signaling. Similar results were found upon repeating the RNA-seq analysis with sorted cells with CD4$^+$ CD44$^+$ CXCR5^{hi} PD-1^{hi} gating strategy (data not shown).

Type I IFN Signaling Aids in Th1 Cell Differentiation In Vivo, at the Expense of that of Tfh Cells

Our RNA-seq data indicate that there is an increase in STAT1-mediated transcription in the absence of STAT3. To determine

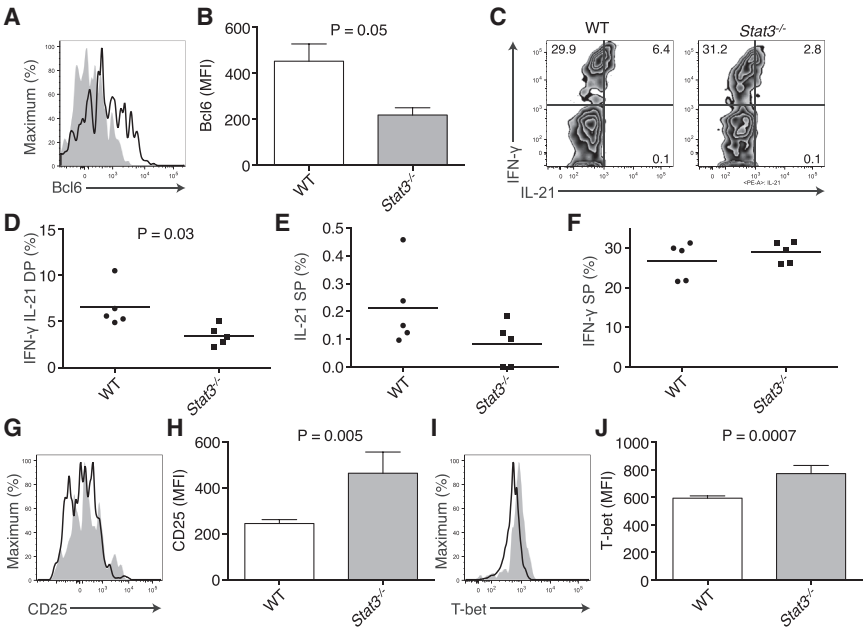


Figure 3. Tfh Cells Deficient in STAT3 Are Less Tfh-like Than Controls, with Characteristics of Th1 Cells

(A and B) Flow cytometry plot (A) and quantification (B) of Bcl6 protein expression in Ly6c^{lo}PSGL-1^{lo}CXCR5^{hi} cells at 5 dpi (WT black line, *Stat3*$^{fl/fl}$*Cd4*^{cre} [*Stat3*$^{-/-}$] shaded gray).

(C–F) Flow cytometry plots (C) and population quantification (D–F) of IL-21 and IFN-γ-expressing cells of the CD4^{+} CD44^{+} CXCR5^{hi} population at 8 dpi (SP, single positive; DP, double positive).

(G and H) Flow cytometry plot (G) and quantification (H) of CD25 protein expression in Ly6c^{lo} PSGL-1^{lo} CXCR5^{hi} cells at 5 dpi.

(I and J) Flow cytometry plot (I) and quantification (J) of T-bet expression in Ly6c^{lo} PSGL-1^{lo} CXCR5^{hi} cells at 5 dpi.

(A–J) Representative of at least two experiments, with at least three animals per genotype per experiment. Statistics conducted with Student's unpaired t test. + SEM (A–D). See also Figure S3.

the role of STAT1 in determining Tfh cell differentiation, we obtained a validated STAT1 small hairpin RNA vector and retrovirally transduced viral epitope GP$_{66}$-specific SMARTA transgenic (Stg) CD4^{+} T cells with it (Choi et al., 2013; Oxenius et al., 1998), and in parallel, with a control vector. Upon transduction, there was substantial silencing of STAT1 (79%, Figures S5A and S5B), and a large reduction in clonal expansion of transduced cells (Figure S5C). We found a slight but reproducible reduction in T-bet expression after transfer into WT congenic hosts (Figure S5D), which was not surprising given that STAT4 has also been shown to drive T-bet expression (Nakayamada et al., 2011). Despite differences in clonal expansion, there were no differences in Tfh cells or Bcl6 expression at 8 dpi (Figures S5E and S5F). It is possible that the use of TCR transgenic T cells nullifies the Tfh cell phenotypes that would otherwise be seen in a polyclonal repertoire (Tubo et al., 2013); however, in line with our data analyzing the polyclonal population (Figures 1A and 1B), we found that STAT3-deficient Stg^{+} cells also had a deficiency in the Tfh cell subset at 8 dpi (Figure S5G). Similar to polyclonal STAT3-deficient T cells, they had no significant differences in Bcl6 protein at 8 dpi (Figure S5H), but there was a marked increase in T-bet expression (Figure S5I). Thus, despite the increase in STAT1-mediated gene transcription in STAT3-deficient T cells, Tfh cell phenotypes were modulated by specific STAT1 signaling cytokines in addition to general STAT1 signaling, because this transcription factor is also known to operate downstream of Tfh-cell inducing cytokines (i.e., IL-6).

Our RNA-seq data suggested a role for both type I and type II IFN signaling in promoting the changes in gene transcription we observed (Figure S4G). Previous work has found that IFN-γ induces Tfh cell formation, and that in the absence of its receptor, *Roquin*$^{san/san}$ lupus-prone mice are rescued from their disease phenotype with reductions in Tfh cell numbers, GCs, and formation of autoantibodies (Lee et al., 2012). To ascertain the effect of IFN-γ on Tfh cell differentiation in our model, we blocked this cytokine by using the XMG1.2 neutralizing antibody (Abrams et al., 1992). Despite a large reduction in splenocyte numbers and T-bet expression upon IFN-γ blockade (Figures S5J and S5K), we found that there was only a marginal, and insignificant, increase in Tfh cell percentages and Bcl6 expression at 8 dpi (Figures S5L and S5M), without an effect on GC B cell percentages (Figure S5N). Thus, despite its role in driving Tfh cells in the *Roquin*$^{san/san}$ autoimmunity model, IFN-γ does not appear to have a large effect on the differentiation of Tfh cells following acute LCMV infection.

We then examined the effects of type I IFNs on Tfh cell differentiation. Type I IFNs are induced following LCMV infection and they regulate STAT1- and IRF7-mediated gene expression (Malmgaard et al., 2002). To determine whether T cell-intrinsic type I IFN signaling is responsible for suppressing Tfh cell formation in favor of Th1 cell differentiation, we crossed *Ifnar1*$^{-/-}$ mice to Stg^{+} mice, and transferred *Ifnar1*$^{-/-}$ Stg^{+} CD4^{+} T cells into infected WT C57BL/6 animals. As previously described (Havenar-Daughton et al., 2006; Way et al., 2007), the expansion of *Ifnar1*$^{-/-}$ Stg^{+} CD4^{+} T cells was severely compromised at 8 dpi (data not shown), indicating an effect on CD4^{+} T cell proliferation and differentiation. In addition, there was an increase in the percentages of Tfh cells along with increased Bcl6 protein, compared to those mice given WT Stg^{+} cells, but with no effects on T-bet expression (Figures 5A–5C; Figure S5O).

Given these findings, we wondered whether type I IFN signaling could actively suppress Tfh cell differentiation, which could potentially explain the propensity of *Stat3*$^{fl/fl}$*Cd4*^{cre} CD4^{+} T cells to develop a Th1-like phenotype. Indeed, upon type I IFN treatment of LCMV epitope-stimulated Stg^{+} WT CD4^{+} T cells, there was an increase in expression of CD25 and phospho-STAT5 (p-STAT5) downstream of IL-2 stimulation (Figure 5D). We found this effect to be dependent on IL-2 and reversed upon treatment with an IL-2 blocking antibody (Figure 5E), suggesting that direct type I IFN signaling might promote IL-2 responsiveness and Th1 differentiation during viral infection in vivo (Johnston et al., 2012; Nurieva et al., 2012; Oestreich

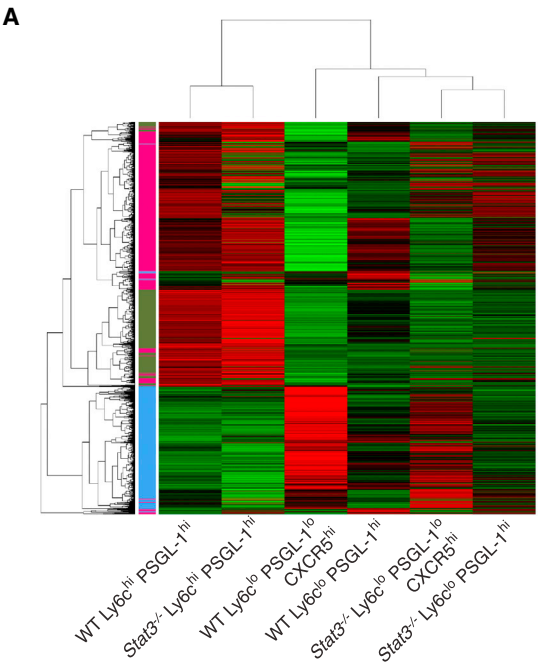


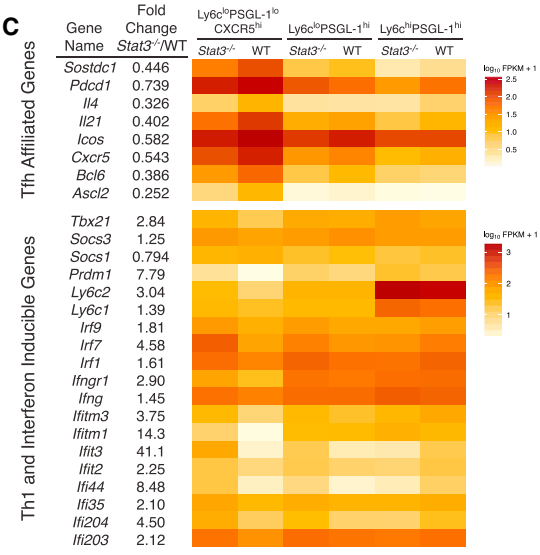
Figure 4. STAT3-Deficient Tfh Cells Have a Th1-like Transcriptome

(A and B) Expression analysis of Ly6c^{lo} PSGL-1^{lo} CXCR5^{hi}, Ly6c^{lo} PSGL-1^{hi}, and Ly6c^{hi} PSGL-1^{hi} cells from *Stat3*$^{fl/fl}$*Cd4*^{cre} (*Stat3*$^{-/-}$) and *Stat3*$^{fl/fl}$ *cre*-negative (WT) mice. (A) Unbiased K-means clustering and dendrogram based on differential gene expression of the three WT populations (see Figure S4B). The bar on the left denotes from which cluster genes come based on Figure S4B. The blue cluster is most associated with WT Tfh cells, green with both WT Th1 cell (PSGL-1^{hi}) populations, and magenta with WT terminal effector cells. Reduced expression is in green with induced expression in red. (B) List of differentially expressed genes from the IPA T helper cell differentiation pathway, with those in red associated with Th1 cells and those in green with Tfh cells. (C) Tfh-cell associated and Th1 and IFN-inducible genes upregulated in STAT3-deficient Tfh cells. Fold changes listed as the ratio of *Stat3*$^{fl/fl}$*Cd4*^{cre} (*Stat3*$^{-/-}$) to WT Tfh cell expression. See also Figure S4 and Table S1.

B

	Gene	Fold Change
Upregulated	*Cd86*	8.51
	Il10ra	8.47
	Il10	6.77
	Cd80	3.86
	Ifngr1	2.90
	Tbx21	2.84
	Il2ra	2.68
	Foxp3	2.45
	Il12rb2	2.39
	Tnfrsf1b	2.20
	Il4r	1.83
Downregulated	*Icos*	1.72
	Il6st	1.82
	Cxcr5	1.84
	Il21	2.48
	Hla-dob	2.49
	Bcl6	2.89
	Il4	3.06

C



et al., 2012). It is possible that T cells deficient in STAT3 are more sensitive to type I IFN and IL-2 signaling, because this transcription factor has been shown to suppress STAT5 signaling (Oestreich et al., 2012). In accordance with this hypothesis, STAT3-deficient cells had IL-2-dependent enhanced expression of CD25 with peptide stimulation and IFN-β treatment compared to WT cells (Figure 5E). STAT3 binding in the *Bcl6* locus occurs in conditions with low IL-2, whereas high amounts of IL-2 allow STAT5 to outcompete STAT3 for binding sites, hampering *Bcl6* expression (Oestreich et al., 2012). To determine how type I IFNs might affect STAT binding to the *Bcl6* locus, we used chromatin immunoprecipitation-qPCR (ChIP-qPCR) to determine binding of STAT1, STAT3, and STAT5 in the presence of IL-6 or type I IFNs. We used previously confirmed (*Bcl6 4*) and newly generated qPCR primer sets surrounding STAT-binding sites (Figure 5F) (Oestreich et al., 2012). While STAT3 bound the *Bcl6* locus in the presence of IL-6, STAT5 bound robustly in the presence of type I IFNs in all primer sets tested (Figure 5G), with little binding of STAT1 in either context. This suggests that type I IFN-induced IL-2 signaling leads to STAT5 binding at the expense of STAT3. Thus, in the absence of STAT3 following acute viral infection, IL-2 and STAT5 signaling is likely dominant, leading to a reduction in Tfh cell differentiation.

Type I IFN Blockade Partially Rescues the *Stat3*$^{fl/fl}$*Cd4*^{cre} Tfh Cell, GC B Cell, and Pathogen-Specific Antibody Phenotypes

Although type I IFNs appear to insulate against Tfh cell in favor of Th1 cell differentiation during viral infection, we hypothesized that this effect might be exacerbated in the absence of STAT3 signaling. To determine whether blockade of type I IFNs could rescue the Tfh cell defect in LCMV-infected mice bearing STAT3-deficient CD4$^+$ T cells, we used the MAR1-5A3 (MAR1) IFN-αβR blocking antibody (Sheehan et al., 2006). Unlike the *Ifnar1*$^{-/-}$ CD4$^+$ T cells, pretreating WT and *Stat3*$^{fl/fl}$*Cd4*^{cre} mice with the MAR1 antibody at a dosage of 600 μg per mouse did not compromise expansion of CD44$^+$ or GP66$^+$ CD4$^+$ T cells following viral challenge (data not shown). Upon pretreatment with MAR1, *Stat3*$^{fl/fl}$*Cd4*^{cre} mice and their WT littermates had substantial increases in Ly6c^{lo} PSGL-1^{lo} Tfh cells, as well as Ly6c^{lo} PSGL-1^{hi} Th1 cells, while there was a decrease in Ly6c^{hi} PSGL-1^{hi} Th1 cells among both GP66$^+$ and total CD44$^+$ cells (Figures 6A–6E; Figures S6A–S6E). Notably, the *Stat3*$^{fl/fl}$*Cd4*^{cre} mice treated with MAR1 had similar Ly6c^{lo} PSGL-1^{lo} cell percentages to WT IgG treated mice, suggesting that STAT3 and type I IFNs act as mutually

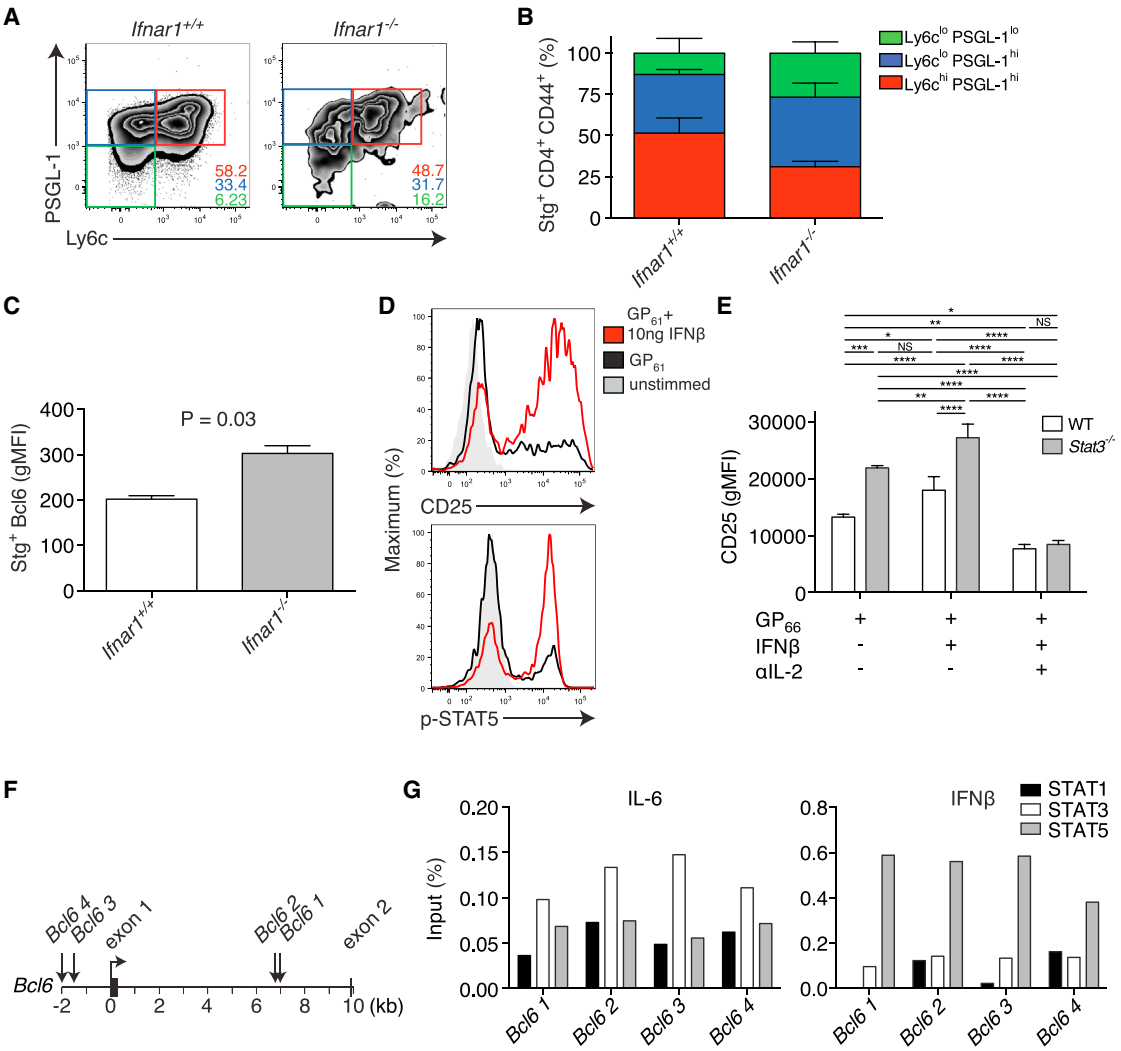


Figure 5. Type I IFNs Inhibit Tfh Cell Differentiation

(A) Flow cytometry plots of *Ifnar1*$^{+/+}$ and *Ifnar1*$^{-/-}$ CD4$^+$ CD44$^+$ Stg$^+$ cells gated on Ly6c and PSGL-1 at 8 dpi.

(B) Ly6c and PSGL-1 populations, gated as in (A).

(C) Bcl6 protein expression in *Ifnar1*$^{+/+}$ and *Ifnar1*$^{-/-}$ Stg$^+$ cells.

(D) Flow cytometry plots of CD25 and p-STAT5 expression of WT Stg$^+$ cells, either unstimulated (unstimmed) or stimulated with the cognate LCMV peptide GP_{61} for 4 days, or with this peptide + 10 ng IFN-β for 4 days. p-STAT5 samples were stimulated with IL-2 for 25 min prior to fixation.

(E) CD25 protein expression for WT and *Stat3*$^{fl/fl}$*Cd4*^{cre} (*Stat3*$^{-/-}$) Stg$^+$ T cells stimulated with the cognate LCMV peptide GP_{66} for 3 days, with this peptide + 10 ng IFNβ for 3 days, or with peptide, 10 ng IFN-β, and αIL-2.

(F) Schematic showing the location of primer sets (arrows) used to determine STAT binding in the *Bcl6* locus. Tic marks are 1 kb and indicate the distance from the transcription start site.

(G) ChIP-qPCR of STAT1, STAT3, or STAT5 for STAT binding within the *Bcl6* locus on WT Stg$^+$ T cells treated with 10 ng/mL IL-6 or IFN-β.

(A–D) Representative of at least three experiments, with at least three animals per genotype per experiment.

(E and G) Representative of at least two experiments. Statistics conducted with Student's unpaired t test or one-way ANOVA with Tukey posttest; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (E). + SD (C and E). See also Figure S5.

repressive insulators for the differentiation of Tfh and Th1 cells, respectively (Figures 6A–6C; Figures S6A–S6C). The WT MAR1-treated mice also had a remarkable increase in Tfh cells beyond that of treated *Stat3*$^{fl/fl}$*Cd4*^{cre} mice, suggesting that STAT3 might have direct effects on Tfh cell differentiation other than insulating against type I IFNs. To corroborate these data, we found at 8 dpi a significant increase in Bcl6 protein expression in both *Stat3*$^{fl/fl}$*Cd4*^{cre} and WT GP_{66}^+ and total CD4$^+$ CD44$^+$ T cells upon MAR1 treatment, compared to a lack of differential expression between untreated *Stat3*$^{fl/fl}$*Cd4*^{cre} and WT mice (Figure 6F; Figure S6F). CXCR5 expression was unaffected upon treatment in GP_{66}-specific T cells, although increased among total WT CD44$^+$ cells (Figure 6G; Figure S6G). We found a repeatable, albeit insignificant, reduction in IFN-γ production upon MAR1 treatment (Figure 6H). The effects on IL-21, however, were more variable, with a possible increase in IL-21 SP

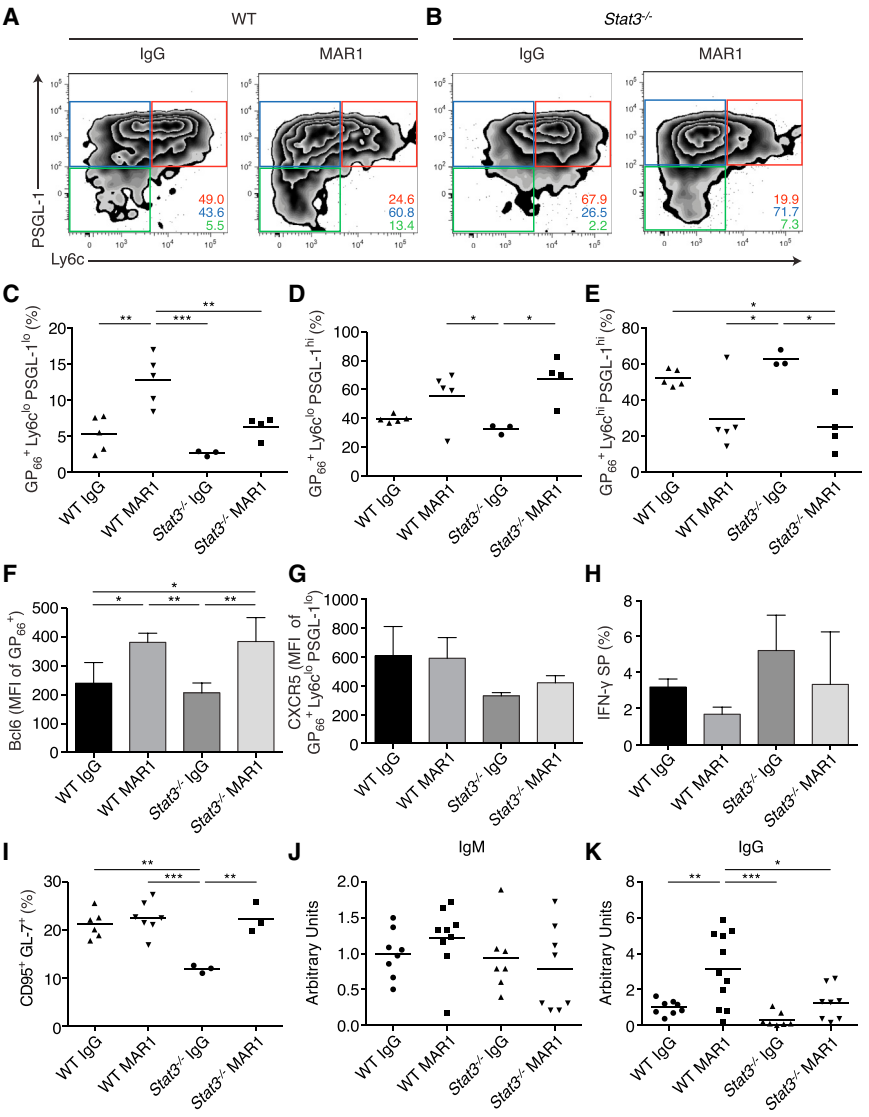


Figure 6. Inhibition of Type I IFN Signaling Partially Rescues STAT3-Deficient Tfh Cell Phenotypes

(A and B) Flow cytometry plots of WT (A) or *Stat3*$^{fl/fl}$*Cd4*^{cre} (*Stat3*$^{-/-}$) (B) CD4$^+$ CD44$^+$ GP$_{66}^+$ Ly6c and PSGL-1 expressing cells, with treatment of the MAR1 blocking antibody or isotype control (IgG).

(C–E) Comparisons of percentages of GP$_{66}^+$ Ly6c and PSGL-1 populations between isotype and MAR1-treated WT and *Stat3*$^{fl/fl}$*Cd4*^{cre} (*Stat3*$^{-/-}$) mice, using the gating in (A).

(F) Bcl6 MFI of GP$_{66}^+$ cells.

(G) CXCR5 MFI of GP$_{66}^+$ Ly6c^{lo} PSGL-1^{lo} cells.

(H) IFN-γ SP cells gated on CD4$^+$ CD44$^+$ CXCR5$^+$.

(I) GC B cell percentages gated on B220$^+$ IgD^{lo} CD95$^+$ GL-7$^+$ cells.

(J and K) Serum concentrations of LCMV-specific IgM (J) and IgG (K).

(A–K) Representative of two to three experiments, with at least three animals per condition per genotype. (J) and (K) are pooled from two separate experiments, where serum concentrations were normalized to WT IgG treated. Statistics conducted with one-way ANOVA with Tukey posttest. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. + SEM (F–H). See also Figure S6.

and IL-21 IFN-γ DP cells in both WT and *Stat3*$^{fl/fl}$*Cd4*^{cre} mice (Figures S6H and S6I).

To analyze the downstream effect of increased Tfh cells in the MAR1-treated mice, we studied GC B cell percentages and pathogen-specific antibody responses. We found that, unlike the effect on Tfh cells, MAR1 had a minimal effect on GC B cell percentages in WT mice. However, in the animals containing STAT3-deficient T cells, GC B cells were rescued to WT percentages (Figure 6I). In line with this finding, whereas effects on IgM were no different, pathogen-specific IgG antibody responses were rescued to the concentrations of WT control animals (Figures 6J and K). This suggests that type I IFNs play an inhibitory role in plasmablast and plasma cell responses in addition to deterring the GC response. We also found a moderate increase in SOCS3 protein expression in the STAT3-deficient Tfh cells (Figure S6J), in agreement with our qPCR data (Figure S4I). This was reduced by MAR1 treatment (Figure S6J), suggesting that type I IFNs elicit the increase in SOCS3 expression found in the *Stat3*$^{fl/fl}$*Cd4*^{cre} mutant.

These data reveal a fine balance in signaling pathways following acute viral infection with a requirement for STAT3 for Tfh cell differentiation and type I IFNs to promote that of the Th1 response.

DISCUSSION

We found that STAT3 is required for Tfh cell differentiation and function during acute viral infection. Mice with Tfh cells that lack STAT3 display impaired GC B cell and plasmablast formation leading to the absence of antigen-specific antibody responses, with promotion of Th1 cell differentiation mediated by type I IFN signaling. Thus, there exists in vivo tight cytokine regulation of Tfh versus Th1 cell differentiation.

STAT3 has been previously noted to aid in Tfh cell differentiation following immunization with a nominal antigen (Nurieva et al., 2008). In a like manner, it has been shown to make a transient contribution to Tfh cell development following LCMV infection (Choi et al., 2013), with the downstream effects of this process in terms of GC responses consequently not analyzed (Choi et al., 2013). However, we found a defect in the development of STAT3-deficient polyclonal and Stg$^+$ Tfh cells at day 8 following viral challenge, indicating that STAT3 affects Tfh cell differentiation later than day 3. This earlier published work also demonstrated that STAT1 aids STAT3 in transient promotion of Tfh cell differentiation (Choi et al., 2013). Our finding that type I IFNs perturb Tfh cell development in the absence of STAT3 suggests that these cytokines utilize STAT1 in a cytokine-specific manner in this process. Indeed, we did not see promotion of

Tfh cells upon silencing of STAT1; so, it is possible that while STAT1 can deter Tfh cell differentiation through signal transduction mediated by type I IFNs, it might also promote Tfh cell differentiation through other cytokines (i.e., IL-6).

There are several possible mechanisms for type I IFN and STAT3 cross-repression that can explain our findings, given the complex regulatory network that involves competition for binding partners, binding sites, and upregulation of repressive SOCS proteins. For example, STAT3 has the capacity to bind to the same consensus region as STAT1, potentially blocking its transcription and vice versa (Wang et al., 2011). The N-terminal domain of STAT3 also has been shown to inhibit type I IFN signaling following viral infection of mouse embryonic fibroblasts through sequestration of STAT1. However, we did not find promotion of Tfh cell differentiation in STAT1-silenced Stg^+ T cells following viral infection. STAT3 and type I IFN cross regulation also could occur via their respective downstream signaling attenuators, SOCS1 and SOCS3. For example, in the absence of STAT3, a lack of either could lead to an increase in STAT1 or STAT5 phosphorylation, reminiscent of that seen in $CD8^+$ T cells in the absence of STAT3 (Cui et al., 2011). However, this mechanism also seems unlikely, as we observe an increase in the expression of SOCS1 and SOCS3 transcripts and SOCS3 protein expression in the $Stat3^{fl/fl}Cd4^{cre}$ in a type I IFN-dependent manner.

Another possible mechanism for type I IFN and STAT3 cross-repression, and one apparent from our results, is through type I IFN promotion of CD25 expression and subsequent STAT5 phosphorylation. In the absence of STAT3, CD25 expression is increased, and upon stimulation of WT STg^+ cells with their cognate peptide and type I IFNs, its expression is also increased, accompanied by enhanced p-STAT5, in an IL-2-dependent manner. STAT5 competes with STAT3 for consensus DNA binding sites, including at the *Bcl6* locus (Oestreich et al., 2012). We found that STAT5 outcompeted STAT3 for binding at the *Bcl6* locus in the presence of type I IFNs. STAT3-deficient Tfh cells appear to have an increase in *Blimp-1* expression, and STAT5 has also been shown to upregulate *Blimp-1*, potentially inhibiting Tfh cell differentiation through cross-repression of Bcl6 and with promotion of Th1 cell differentiation (Johnston et al., 2009, 2012; Nurieva et al., 2012; Oestreich et al., 2012). Thus, increased IL-2 signaling via type I IFNs likely upregulates Blimp-1 and deters Bcl6-mediated Tfh cell differentiation (Johnston et al., 2012; Nurieva et al., 2012; Oestreich et al., 2012). In the presence of STAT3, Tfh cell differentiation was promoted upon type I IFN blockade during acute viral infection. Therefore, it is likely that STAT3 plays multiple roles, one as an insulator against type I IFNs and another as an enhancer of Tfh cell differentiation. Likewise, type I IFNs might also play dual roles, by both promoting Th1 cell differentiation through STAT1 induction of *Tbx21* and Tfh cell differentiation through upstream (or indirect) induction of autocrine IL-2, subsequently leading to STAT5 activation and direct competition with STAT3 for binding sites in the *Bcl6* locus.

Patients with dominant-negative STAT3 mutations have characteristic decreases in circulating "Tfh-like" cells in their blood, as well as lower serum antigen-specific IgG concomitant with increased IgE (Ma et al., 2012). Many of these patients suffer recurrent infections from *Candida albicans* and *Staphylococcus aureus* due to a scarcity of Th17 cells, with STAT3 signaling necessary for the development of this subset (de Beaucoudrey et al., 2008; Ma et al., 2008; Milner et al., 2008; Renner et al., 2008). Given these findings and that such patients have deficiencies in memory T cells, it is prudent to further characterize the T and B cell intrinsic components of STAT3 signaling in developing long term immunity. Patients with the autoimmune syndrome systemic lupus erythematosus (SLE) have high titers of autoantibodies and an abundance of Tfh cells (Craft, 2012); thus, blockade of STAT3 signaling might be therapeutically beneficial, with a decrease in T cell-mediated autoantibody production. Although we show here that STAT3 plays a role in Tfh cell differentiation in acute viral infection, this transcription factor also is required for regulating Th2 type immunity (Stritesky et al., 2011). We and others have shown that in the absence of STAT3, there is a drastic reduction in IL-4 production, with STAT3 important for opening the IL-4 locus to enable STAT6 binding (Stritesky et al., 2011). In Th2 cell-associated infections, IL-4 production by T cells is important for inducing antigen-specific IgE production by plasma cells. Given the importance of STAT3 in promoting Th2 cell type immunity, it will be critical to explore the effects of $CD4^+$ T cell STAT3 signaling in exacerbating allergic disease such as asthma. STAT3 inhibitors are in development, and this could be a useful therapeutic for those who suffer from Tfh-cell mediated autoimmune diseases such as SLE, or those with allergic illnesses, given the effect of STAT3 on IL-4 production.

EXPERIMENTAL PROCEDURES

Mice and LCVM Infection

Mice were housed in specific pathogen-free conditions at the Yale School of Medicine (New Haven, CT). C57BL/6 (B6) mice were purchased from the National Cancer Institute (Bethesda, MD), with other strains provided as noted: $Stat3^{fl/fl}$ (B6.129S1-*Stat3tm1Xyfu*/J) mice from Xin-Yuan Fu at the Indiana University School of Medicine, $Cd4^{cre}$ (B6.Cg-*Tg(Cd4-cre)1Cwi/BfluJ*) from Christopher Wilson at the University of Washington in Seattle, SMARTA Tg (*Tg(TcrLCMV)1Aox*) from Hans Hengartner at the University of Zürich in Switzerland, and $Ifnar1^{-/-}$ ($Ifnar1^{tm1Agt}$) from Michel Aguet at the University of Zürich in Switzerland. All were used at 6–8 weeks of age. The Institutional Animal Care and Use Committee of Yale University approved all procedures involving mice. They were infected with LCMV Armstrong by intraperitoneal (i.p.) injection of 2×10^5 PFU per mouse.

Antibodies for Flow Cytometry and Cell Sorting

These protocols are described in detail in the Supplemental Experimental Procedures.

Microscopy

These protocols are described in detail in the Supplemental Experimental Procedures.

ELISA for Antibodies to LCMV

Anti-LCMV Ab was measured by ELISA with sonicated cell lysate from LCMV-infected BHK-21 cells as capture Ag. Ninety-six-well Polysorp microtiter plates (Nunc) were coated overnight with lysate in PBS. AP-conjugated goat anti-mouse IgM, IgG, and IgG2a secondary Abs were used for detection (Southern Biotech). ODs were converted to units based on standard curves with sera from WT mice infected with LCMV (Softmax Pro 3.1 software; Molecular Devices).

RNA-Seq

Sorted Tfh cells were lysed with their RNAs isolated with the miRNeasy kit (QIAGEN). Samples were sequenced on an Illumina HiSeq 2000 with 75-bp paired-end reads. FASTQ format sequencing reads were aligned to the MM9 mouse genome using TopHat version 2.0.6 and Bowtie version 1.3.0.

Reads were compared between samples with Cufflinks version 2.0.2. Heatmaps were made with CummeRbund version 2.0.0. Quantitative real-time PCR was used to confirm expression of RNA transcripts. IPA Ingenuity® Systems was used to analyze deregulated pathways and potential upstream regulators.

Quantitative PCR

These protocols are described in detail in the Supplemental Experimental Procedures.

Retroviral Transduction and Cell Transfer

These protocols are described in detail in the Supplemental Experimental Procedures.

Immunoblot

These protocols are described in detail in the Supplemental Experimental Procedures.

Chromatin Immunoprecipitation

These protocols are described in detail in the Supplemental Experimental Procedures.

Type I IFN, IFN-γ, and IL-2 Blockade

For type I IFN blockade, WT or *Stat3*$^{fl/fl}$*Cd4*^{cre} mice were treated at day −1 with 600 μg of MAR1-5A3 IFN-αβR blocking antibody or IgG1 isotype control antibody (both from Leinco Technologies) in PBS via i.p. injection (Sheehan et al., 2006), before infection with LCMV Armstrong at day 0. For IFN-γ blockade, WT mice were treated at day 0 and day 3 after LCMV Armstrong infection with 250 μg of XMG1.2 IFN-γ blocking antibody or PBS vehicle control (Abrams et al., 1992). IL-2 blocking antibody (JES6-1A12) was used in vitro at a concentration of 10 μg/mL (Abrams et al., 1992).

Statistics

Data were analyzed with the Student's unpaired t test or with one-way ANOVA with Tukey posttest for multiple comparisons with Prism 6®. The number of asterisks represents the degree of significance with respect to p values, and the p value is presented within each figure or figure legend.

ACCESSION NUMBERS

The GEO accession number for the RNA-Seq data reported in this paper is GSE55596.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.02.005.

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